THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

U.S. Environmental Protection Agency

ETV Joint Verification Statement

TECHNOLOGY TYPE:	**IMMUNOASSAY TEST KITS**
APPLICATION:	**DETECTING ANTHRAX, BOTULINUM TOXIN, AND RICIN**
TECHNOLOGY NAME:	**Enzyme-Linked Immunosorbent Assay**

COMPANY:	**Tetracore, Inc.**		
ADDRESS:	**11 Firstfield Road, Suite C**	**PHONE:**	**301-258-7553**
	Gaithersburg, MD 20878	**FAX:**	**301-258-9740**
WEB SITE:	**www.tetracore.com/**		
E-MAIL:	**tobrien@tetracore.com**		

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative or improved environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies. Information and ETV documents are available at www.epa.gov/etv.

ETV works in partnership with recognized standards and testing organizations, with stakeholder groups (consisting of buyers, vendor organizations, and permitters), and with individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The Advanced Monitoring Systems (AMS) Center, one of six verification centers under ETV, is operated by Battelle in cooperation with EPA's National Exposure Research Laboratory. The AMS Center has recently evaluated the performance of immunoassay test kits used to detect anthrax, botulinum toxin, and ricin. This verification statement provides a summary of the test results for the Tetracore, Inc., enzyme-linked immunosorbent assay (ELISA).

VERIFICATION TEST DESCRIPTION

The ability of the Tetracore ELISA to individually detect various concentrations of anthrax spores, botulinum toxin, and ricin was evaluated between January 14 and April 23, 2004, by analyzing performance test (PT) and drinking water (DW) samples. PT samples included deionized (DI) water fortified with either the target contaminant, an interferent, both, or only a cross-reactive species. In addition to the PT and DW samples analyzed, method blank (MB) samples consisting of DI water also were analyzed to confirm negative responses in the absence of contaminants and to ensure that no sources of contamination were introduced during the analysis procedures. Verification test results showed how effective the Tetracore ELISA was at detecting the presence of each contaminant at several concentration levels, the consistency of the responses, and the susceptibility of the Tetracore ELISA to selected interferents and cross-reactive species. In most cases, three replicates of each PT and DW sample were analyzed to evaluate the reproducibility of the Tetracore ELISA results. Approximately 120 liters (L) of four DW samples were collected from geographically distributed municipal sources located in Florida (FL), New York (NY), Ohio (OH), and California (CA). These samples were dechlorinated with sodium thiosulfate, and then 100 L of each sample were concentrated using an ultra-filtration technique to a final volume of 250 milliliters (mL). Each DW sample (non-concentrated and concentrated) was analyzed without adding any contaminant, as well as after fortification with individual contaminants at a single concentration level to evaluate the effect of the DW matrix on the performance of the Tetracore ELISA. During the anthrax spore PT sample analysis, the lowest detectable concentration of the Tetracore ELISA was shown to be much higher than claimed by the vendor. Therefore, two preparations of spores were analyzed to further investigate these results. The two preparations included spores prepared at Battelle and preserved in a solution of water and phenol and spores prepared at Dugway Proving Ground and stored in spent culture media. Most of the samples analyzed were made from the Battelle-prepared, phenol-preserved spores. The other preparation was used to determine if the phenol preservation or the preparation technique was negatively affecting the sensitivity of the Tetracore ELISA. Solutions of vegetative anthrax cells also were analyzed to determine the sensitivity of the Tetracore ELISA to vegetative anthrax cells.

QA oversight of verification testing was provided by Battelle and EPA. Battelle QA staff conducted a technical systems audit and a data quality audit of 10% of the test data. This verification statement, the full report on which it is based, and the test/QA plan for this verification are all available at www.epa.gov/etv/centers/center1.html.

TECHNOLOGY DESCRIPTION

The following description of Tetracore ELISA was provided by the vendor and was not subjected to verification in this test.

The antigen-capture Tetracore ELISA detects antigens in samples by capturing them between a sandwich of antibodies. The immunosorbent assay uses immunological reagents to identify antibodies. The Tetracore ELISA can be read qualitatively (visually) and recorded by hand or quantitatively (using a photometer that measures and prints out the optical density of fluid samples in the microplate). Readings were made qualitatively during this verification test. To perform a test, positive and negative capture antibody reagents are applied to alternating wells of a 96-well plate, where they are passively adsorbed. If the target antigen is present in a sample, it will bind to the reagent. A detector antibody forms the top of the sandwich and binds to any antigen in the sample after it is captured. The conjugate, to which the enzyme is covalently bound, is the third reagent added; and it binds to the detector antibody. The substrate, added after the conjugate, contains 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate), which, in the presence of horseradish peroxidase, changes to a bright green. The amount of color change is directly proportional to the amount of horseradish peroxidase present, which correlates to the amount of antigen (target contaminant) bound in the sandwich. The color change confirms the “capture” of antigen by the antibody reagents. For 48 samples, the process takes approximately 5 hours. The Tetracore ELISA includes two 96-well plates, dilution buffer, wash buffer, and the appropriate reagents needed for the analysis. The 96-well microplate is 12.5 centimeters (cm) by 8 cm. One Tetracore ELISA (positive and negative coated wells) costs $400.

VERIFICATION OF PERFORMANCE

The tables below summarize the performance of the Tetracore ELISA in detecting anthrax, botulinum toxin, and ricin.

Anthrax Summary Table

Parameter		Sample Information	Actual Fortified Anthrax Concentration[a]	Positive Results Out of Total Replicates
Qualitative contaminant results	Contaminant-only PT samples	Battelle-prepared, phenol-preserved spores	8×10^8 spores/mL	3/3
			8×10^7 spores/mL	3/3
			8×10^6 spores/mL	3/3
			8×10^5 spores/mL	0/3
		Vegetative cells	3×10^5 colony-forming units (cfu/mL)	3/3
			3×10^4 cfu/mL	3/3
			3×10^3 cfu/mL	0/3
			3×10^3 cfu/mL	0/3
		Dugway-prepared spores	8×10^6 spores/mL	0/3
			8×10^5 spores/mL	0/3
			8×10^4 spores/mL	0/3
			8×10^3 spores/mL	0/3
	Interferent PT samples	230 mg/L Calcium (Ca) 90 mg/L Magnesium (Mg)	8×10^7 spores/mL[b]	3/3
		2.5 mg/L humic acid 2.5 mg/L fulvic acid	1×10^8 spores/mL[b]	3/3
		Humic acid and fulvic acid	2×10^6 spores/mL[b]	6/6
		Ca and Mg	2×10^6 spores/mL	0/6
	DW samples	Concentrated CA	5×10^7 spores/mL[b]	3/3
		Concentrated NY	5×10^7 spores/mL[b]	3/3
		Unconcentrated DW	2×10^6 spores/mL	0/24
	Cross-reactivity	1×10^4 spores/mL *Bacillus thuringiensis*	unspiked	0/3
False positives		No false positives resulted from the analysis of the interferent, DW, or cross-reactivity samples. However, two humic and fulvic acid samples, spiked at concentrations below what was detectable in DI water, generated positive results. *Bacillus thuringiensis* was prepared at concentrations much lower than the lowest detectable concentration of *Bacillus anthracis*. Therefore, negative results with these samples do not necessarily indicate a lack of cross-reactivity.		
False negatives		No false negative results were generated for the analysis of interferent or DW samples spiked with detectable levels of anthrax. Tetracore ELISA was not able to detect anthrax at the vendor-stated limit of detection (LOD), but was able to at much higher concentrations. All of the unconcentrated DW samples and six Ca and Mg samples were spiked at concentrations less than detectable and, therefore, were, as expected, negative.		
Consistency		100% (47 out of 47) of the results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		
Lowest detectable concentration		8×10^6 spores/mL - Battelle prep (vendor-stated LOD: 2×10^4 spores/mL); 3×10^4 cfu/mL - vegetative anthrax (no vendor-stated LOD); the Dugway preparation of spores was not detectable at concentrations up to 8×10^6 spores/mL		

[a] The uncertainty of the enumeration technique is approximately 15%.
[b] Battelle-prepared, phenol-preserved spores.

Botulinum Toxin Summary Table

Parameter		Sample Information	Botulinum Toxin Concentration (mg/L)	Positive Results Out of Total Replicates
Qualitative contaminant positive results	Contaminant-only PT samples	Type A	0.004	0/3
			0.02	3/3
			0.04	3/3
			0.2	3/3
		Type B	0.004	2/3
			0.02	0/3
			0.04	1/3
			0.2	3/3
			0.3	1/3
	Interferent PT samples	Ca and Mg	0.04	3/3 Type A 6/6 Type B
		Humic acid and fulvic acid	0.04	1/3 Type A 3/6 Type B
	DW samples	Concentrated DW	0.04	6/6 Type A 12/12 Type B
		Unconcentrated DW	0.04	6/6 Type A 12/12 Type B
	Cross-reactivity	0.04 mg/L Lipopolysaccharide	unspiked	0/3
False positives		There were no false positive results for the interferent, DW, or cross-reactivity samples.		
False negatives		Two out of three results were false negative when 0.04 mg/L botulinum toxin Type A was spiked into 2.5 mg/L humic and fulvic acids, and three out of three were false negatives when botulinum toxin Type B was spiked into 0.5 mg/L humic and fulvic acids. There were no false negatives for the spiked DW samples.		
Consistency		With the exception of 2.5 mg/L humic and fulvic acids spiked with 0.04 mg/L botulinum toxin Type A (1 out of 3 positive), results generated for botulinum toxin Type A were 100% consistent. The DW and interferent samples spiked with botulinum toxin Type B were equally consistent, but the contaminant PT samples containing botulinum toxin Type B generated consistent results in just 2 out of 5 sample sets. Overall, 98% of the results were from sample sets that were either all positive or all negative.		
Lowest detectable concentration		0.02 mg/L (Type A); not clear for Type B because of sporadic results. (vendor-stated LOD for botulinum toxin [non-specific]: 0.004 mg/L)		

Ricin Summary Table

Parameter		Sample Information	Ricin Concentration (mg/L)	Positive Results Out of Total Replicates
Qualitative contaminant positive results	Contaminant-only PT samples	Ricin PT samples	0.0015	0/3
			0.0075	3/3
			0.015	3/3
			0.075	3/3
			15	3/3
	Interferent PT samples	Ca and Mg	0.015	6/6
		Humic acid and fulvic acid	0.015	6/6
	DW samples	Concentrated CW	0.015	12/12
		Unconcentrated DW	0.015	12/12
	Cross-reactivity	0.015 mg/L Lectin from soybean	unspiked	0/3
False positives		No false positive results were generated for ricin in DW or interferent samples.		
False negatives		There were no false negative results for interferent or DW samples spiked with detectable concentrations of ricin.		
Consistency		100% of the results for ricin were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		
Lowest detectable concentration		0.0075 mg/L (vendor-stated LOD: 0.0015 mg/L)		

Other Performance Factors for Anthrax, Botulinum Toxin, and Ricin: A technically trained operator easily performed the Tetracore ELISA analysis. Untrained, non-technical, first-time users would not likely be able to perform the testing because of the need to use a multichannel pipettor, prepare solutions, and read a technical operating procedure. The Tetracore ELISA could be used outside the laboratory without a problem. At times it was difficult to determine whether the color of the sample had changed; no reader was used. Sample throughput was 48 samples in 5 hours.

Original signed by Gabor J. Kovacs 9/13/04
Gabor J. Kovacs Date
Vice President
Energy and Environment Division
Battelle

Original signed by E. Timothy Oppelt 9/21/04
E. Timothy Oppelt Date
Director
National Homeland Security Research Center
U.S. Environmental Protection Agency